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The current state of serum biomarkers of hepatotoxicity

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Abstract

The level of serum alanine aminotransferase (ALT) activity reflects damage to hepatocytes and is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of hepatotoxicity. However, an increase in serum ALT activity level has also been associated with other organ toxicities, thus, indicating that the enzyme has specificity beyond liver in the absence of correlative histomorphologic alteration in liver. Thus, unidentified non-hepatic sources of serum ALT activity may inadvertently influence the decision of whether to continue development of a novel pharmaceutical compound. To assess the risk of false positives due to extraneous sources of serum ALT activity, additional biomarkers are sought with improved specificity for liver function compared to serum ALT activity alone. Current published biomarker candidates are reviewed herein and compared with ALT performance in preclinical and on occasion, clinical studies. An examination of the current state of hepatotoxic biomarkers indicates that serum F protein, arginase I, and glutathione-*S*-transferase alpha (GSTα) levels, all measured by ELISA, may show utility, however, antibody availability and high cost per run may present limitations to widespread applicability in preclinical safety studies. In contrast, the enzymatic markers sorbitol dehydrogenase, glutamate dehydrogenase, paraxonase, malate dehydrogenase, and purine nucleoside phosphorylase are all readily measured by photometric methods and use reagents that work across preclinical species and humans and are commercially available. The published literature suggests that these markers, once examined collectively in a large qualification study, could provide additional information relative to serum ALT and aspartate aminotransferase (AST) values. Since these biomarkers are found in the serum/plasma of treated humans and rats, they have potential to be utilized as bridging markers to monitor acute drug-induced liver injury in early clinical trials.

Keywords: Serum; Alanine aminotransferase (ALT); Glutathione-*S*-transferase alpha (GSTα)

1. Introduction

New drug products require rigorous preclinical and clinical testing and ultimately FDA and EMEA approval prior to being marketed. Many preclinical candidate compounds (PCCs) do not achieve ultimate regulatory approval because of induced organ toxicity. Of the PCCs that are discontinued for organ toxicity, up to half are due to hepatotoxic effects including necrosis, steatosis, cholestasis, proliferation, inflammation, and bile duct hyperplasia. Likewise, the most frequently cited reason for withdrawal of an approved drug is toxicity and often hepatotoxicity is the source of concern (Xu et al., 2004). Assessment of the potential

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for new therapeutics to cause liver injury in humans relies heavily on thorough evaluation of histomorphologic and clinical pathologic endpoints of hepatotoxicity in preclinical species. However, estimates indicate that current preclinical testing regimes successfully correlate to adverse events in human liver in only about 50% of the cases where clinical trials are performed (Olson et al., 2000). Biomarkers that are diagnostic of liver histomorphologic change in preclinical species that might bridge to humans are being evaluated, developed, and improved largely by industrial efforts, which is the subject of this review. Thus, novel biomarkers of liver injury are sought to lower the incidence of false negative results, thereby leading to more accurate prediction of drug-induced liver injury in preclinical and ultimately clinical studies. False positive preclinical indicators of hepatotoxicity are also of concern in pharmaceutical development since they could also impact potential development. Generally, false positive signals show reduced impact upon safety compared to false negative signals.

Ideal attributes of biomarkers of hepatic damage include organ specificity for liver, strong correlation with well-defined hepatic histomorphologic changes, out performance or added information to serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) values, adaptation of screening assays to high throughput modalities that are commercially available, sample accessibility by uninvasive procedures such as blood collection, and in the context of clinical translation, application across key preclinical species to humans, including mouse, rat, dog, and monkey. Once candidate markers are identified, rigorous technical validation or cross-validation and biological qualification is required for wide acceptance by scientific, medical and regulatory authorities. While no single biomarker has yet fully achieved this rigorous level of evaluation, these attributes are a common goal of the pharmaceutical community and regulatory agencies for all safety biomarkers in development. Recommendations for selection of established hepatotoxicity markers appropriate for preclinical screening have been previously discussed (Boone et al., 2005; Ramaiah, 2007). Preclinical assays currently include some combination of serum ALT and AST activities, alkaline phosphatase (ALP) activity, total bilirubin, gamma-glutamyl transferase (GGT) activity, bile acids and/or sorbitol dehydrogenase (SDH) activity (Table 1). Among these assays, some are more specific and/or sensitive than others for liver toxicity. For example, serum AST activity is associated with liver toxicity, but also can be elevated in association with heart and skeletal muscle injury (Burhop et al., 2004; Nathwani et al., 2005). Serum ALT activity is also associated with muscle necrosis. This review will cover the current state of hepatotoxicity biomarkers and their utility in the preclinical and clinical settings. Development of novel candidate hepatotoxicity markers will also be discussed as extensively as possible given current gaps in the published literature (Table 1).

2. ALT is the clinical chemistry gold standard of hepatotoxicity

2.1. Alanine aminotransferase activity is the clinical chemistry gold standard for detection of liver injury

Serum ALT activity level is the most frequently relied upon laboratory indicator of hepatotoxic effects (Amacher, 1998; Amacher, 2002), shows infrequent false negative signals of liver histopathologic injury as well as limited false positive signals and is considered as the gold standard clinical chemistry marker of liver injury. Although the overall clinical utility of serum ALT measurements is exceptional, it does not always correlate well with preclinical histomorphologic data. Thus, additional markers are sought to add information to serum ALT enzymatic signals, especially as bridge biomarkers in early human trials where histopathologic data are usually not available.

ALT plays an important role in amino acid metabolism and gluconeogenesis. ALT and AST catalyze the reductive transfer of an amino group from alanine or aspartate, respectively, to alpha-ketoglutarate to yield glutamate and pyruvate or oxaloacetate, respectively. Damaged hepatocytes release their contents including ALT and AST into the extracellular space. The released enzymes ultimately enter into circulation and thereby increase the serum levels of ALT and AST compared to control subjects. AST is localized in heart, brain, skeletal muscle and liver tissue. ALT is primarily localized to liver, with lower enzymatic activities found in skeletal muscle and heart tissue. Increases in serum ALT and AST enzymatic levels can also arise from extra-hepatic injury, particularly to skeletal muscle. Newer biomarkers of liver injury, with greater specificity to liver, could be used in conjunction with ALT for the safety evaluation of developmental compounds in the pharmaceutical industry.

Serum AST activity is considered a less specific biomarker of liver function compared to ALT activity. AST is released from damaged myocytes as well as hepatocytes. The ratio of serum AST to ALT

Table 1
The abbreviations used are for standard hepatotoxicity assays: alanine aminotransferase activity (ALT), alanine aminotransferase isoform 1 (ALT1), alanine aminotransferase isoform 2 (ALT2), aspartate aminotransferase activity (AST), alkaline phosphatase activity (ALP), gamma-glutamyl transferase activity (GGT); for enzymatic hepatotoxicity assays: sorbitol dehydrogenase activity (SDH), glutamate dehydrogenase activity (GLDH), malate dehydrogenase activity (MDH), purine nucleoside phosphorlyase activity (PNP), paraoxonase-1 activity (PON1); and for ELISA (EIA) hepatotoxicity assays: hydroxyphenylpyruvate dioxygenase (HPD) and glutathione-*S*-transferase α (GSTα). Amino acid (aa) is indicated in biological activity

Biomarker	Cellular localization	Biological activity	Tissue localization	Damage	Detection	Comments
ALT	Cytoplasm and mitochondria, periportal	Amino group reductive transfer from aa	Broad	Necrosis	Colorimetric	Gold standard
ALT1	Cytoplasm and mitochondria	Amino group reductive transfer from aa	Liver restricted	Necrosis	Immuno-enymatic	Possible new standard, isozyme
ALT2	Cytoplasm and mitochondria	Amino group reductive transfer from aa	Skeletal muscle, heart	Necrosis	Immuno-enymatic	Extrahepatic, isozyme
AST	Cytoplasm and mitochondria	Amino group reductive transfer from aa	Broad	Necrosis	Colorimetric	Current standard
Total bilirubin, direct + indirect		Hemoglobin degradation	Indirect (serum), direct (hepatic)	Cholestasis, biliary	Colorimetric	Conventional biliary
ALP	Cell membrane	Phosphatase	Broad	Cholestasis, biliary	Colorimetric	Conventional biliary
GGT	Cell membrane	Gamma-glutamyl transfer	Kidney > liver, pancreas	Cholestasis, biliary	Colorimetric	Conventional biliary
Bile acids		Cholesterol metabolism	Bile duct	Functional	Colorimetric, MS–MS	Total
SDH	Cytoplasm and mitochondria	Sorbitol redox, fructose, NADH	Liver, kidney	Necrosis	Colorimetric	Enzymatic instability
GLDH	Mitochondrial matrix	Amino acid oxidation & urea production	Liver specific > kidney	Necrosis	Colorimetric	Centrilobular enzyme literature cites ↑ with ANIT, CCl4 in rat
PNP	Cytoplasm of endothelial cells, kupffer cells, hepatocytes	Key enzyme in purine salvage pathway	Liver > muscle > heart	Necrosis	Colorimetric	Released into hepatic sinusoids with necrosis literature cites ↑ with galactosamine, endotoxin in rat
MDH	Cytoplasm and mitochondria, periportal	Constituent enzyme of citric acid cycle	Liver > heart > muscle > brain	Necrosis	Colorimetric	Periportal enzyme literature cites ↑ with APAP, TAA in rat
PON1	Cytoplasm, microsomal, nuclear, ER	Protects HDL and LDL from lipid peroxidation; cholesterol metabolism; detoxifies organophosphates	Liver > kidney, brain, lung	Necrosis	Colorimetric	HDL-esterase ↓ with phenobaribital in rats and ↓ in human with chronic liver disease, gene polymorphism
Serum protein F aka HPD	Primarily cytoplasm	Tyrosine catabolism	Liver specific > kidney	Necrosis	EIA, pending	Elevations in human hepatocellular damage
GSTα	Cytoplasm, centrolobular cells	Phase II detox enzyme	Liver specific	Necrosis, prodromal	EIA	↑ with TAA, ANIT, BrB in rats, gene polymorphism
Arginase I	Cytoplasm	Arginine catabolism	Liver	Necrosis	EIA, pending	Literature cites ↑ with TAA in rat

Compound abbreviations in comments include thioacetamide (TAA), 1-naphthylisothiocyanate (ANIT), acetaminophen (APAP), bromobenzene (BrB), carbon tertrachloride (CCl_4), mass spectrometry (MS), and endoplasmic reticulum (ER).

can be used to differentiate liver damage from other organ damage. ALT levels are greater than AST levels in certain types of chronic liver disease such as hepatitis. Muscle necrosis induces an acute spike in AST levels that typically normalizes within several days. Human patients with muscle necrosis induced by extreme exercise but with no known liver disease showed serum AST to ALT activity ratios above 3:1, that were reduced to 1:1 after several days. In the absence of other information these changes in the AST/ALT ratio could be interpreted as evidence of an hepatotoxic event (Nathwani et al., 2005). Skeletal muscle injury can often be verified by elevated serum creatinine kinase, lactate dehydrogenase and/or aldolase activities, yet these markers may not be routinely measured. Thus, routine markers of hepatic function that provide additional information with increased liver specificity, that can be used in conjunction with ALT activity would be valuable bridging markers for PCC evaluation.

2.2. Can the ALT assay be improved?

Since two ALT isozymes have been identified based on molecular structure and tissue specificity, can this information be adapted to create a new and improved ALT assay? Humans express *GPT1* (ALT1) and *GPT2* (ALT2) and these two forms of the enzyme share approximately 70% identity (Yang et al., 2002). *GPT2* mRNA is highly expressed in muscle, fat, brain, and kidney tissues, whereas *GPT1* mRNA is mainly expressed in kidney, liver, fat, and heart tissues (Yang et al., 2002). *GPT2* mRNA is the predominant form in muscle and fat tissues (Yang et al., 2002). Independent reports show that high *GPT2* mRNA levels were detected from human heart and skeletal muscle tissue (Lindblom et al., 2007).

ALT1 immunohistochemical (IHC) reactivity was localized to human hepatocytes, renal tubular epithelial cells and in salivary gland epithelial cells, while ALT2 IHC reactivity was localized to human adrenal gland cortex, neuronal cell bodies, cardiac myocytes, skeletal muscle fibers and endocrine pancreas (Lindblom et al., 2007). Importantly, ALT2 catalyzes alanine transamination indicating that ALT activity is a composite of ALT1 and 2 isozyme activities (Yang et al., 2002). A short (splice) form of ALT2 has also been identified and shows enzymatic activity as well (Lindblom et al., 2007). Discriminating between these two isozymes can therefore be expected to aid in differentiating the tissue source of ALT activity.

2.3. Serum ALT1 and 2 activities can be discriminated in a novel clinical chemistry assay

A novel immunoassay has been shown to discriminate human ALT1 and 2 activities and has been studied in the context of acute drug-induced hepatotoxicity (Lindblom et al., 2007). Immunodepletion of one isozyme allows the specific enzymatic activity of the remaining isozyme to be assayed. This assay can be adapted to a clinical chemistry analyzer for high throughput screening of novel pharmaceutical compounds for preclinical and clinical studies (Lindblom et al., 2007). The depletion approach has not yet been used to discriminate between the two human ALT2 forms.

2.4. ALT1 and 2 are conserved across species

ALT isozymes are conserved across preclinical species and humans. Murine ALT1 and ALT2 are highly homologous to the human proteins (Jadhao et al., 2004) and show similar tissue distribution. The murine homologues share 87% and 93% identity with the human forms of ALT1 and ALT2 respectively. ALT2 has been proposed as a marker of murine hepatic steatosis (Jadhao et al., 2004). Thus, an assay(s) that could discriminate amongst the levels of the three ATL enzyme forms (ALT1, ALT2, and ALT2-small) may add information to composite serum ALT enzymatic activity.

3. Supplemental hepatotoxicity assays that support ALT measurements

Total bilirubin, GGT and alkaline phosphatase (ALP) activities, and bile acids are additional conventional biomarkers of liver function used to supplement serum ALT activity, especially with regard to the differential diagnosis of biliary function.

3.1. Total bilirubin

Total bilirubin is a composite of indirect (non-hepatic) and direct (hepatic) bilirubin. This product of hemoglobin degradation is a marker of hepatobiliary injury, especially cholestasis and biliary effects. In acute human hepatic injury, total bilirubin can be a better indicator of disease severity compared to ALT (Dufour et al., 2001). Bilirubin may also be increased due to non-hepatic causes such as hemolysis. Analysis of indirect compared to direct bilirubin does not necessarily add information in routine assessment when compared to total bilirubin (Stempfel and Zetterstrom, 1955).

3.2. Alkaline phosphatase activity

Alkaline phosphatase (ALP) is associated with cell membranes in multiple tissues, particularly hepatocytes. It hydrolyzes monophosphatases at an alkaline pH. Several alkaline phosphatase isoenzymes (e.g., liver, bone, intestinal, kidney, and placental forms) have been identified in humans and preclinical species. It is primarily a marker of hepatobiliary effects and cholestasis (moderate to marked elevations) (Ramaiah, 2007). In humans with primary biliary cirrhosis, a chronic cholestatic liver disease of unknown etiology, tests reveal elevations of serum alkaline phosphatase activity (20-fold) and GGT activity with or without elevated ALT levels (Nishio et al., 2000). In humans, increased alkaline phosphatase levels have been associated with drug-induced cholestasis (Wright and Vandenberg, 2007). In rats, GGT activity is considered a reliable marker for cholestasis compared to alkaline phosphatase activity.

3.3. Gamma-glutamyl transferase activity

Gamma-glutamyl transferase (GGT) activity is localized to liver, kidney, and pancreas tissues, yet enzyme concentration in liver is low compared to kidney (Antoine et al., 1989). GGT has multiple functions including catalytic transfer of γ-glutamyl groups to amino acids and short peptides (Goldberg, 1980), hydrolysis of GSH to a gamma-glutamyl moiety and cysteinlyglycine in GSH and GSH conjugate catabolism (Csanaky and Gregus, 2005). GGT also contributes to pancreatic transport of amino acids across cell membranes (Sastre et al., 1991), and cleaves the gamma-glutamyl linkage of leukotriene C4 (Heisterkamp et al., 1991). GGT activity is a marker of hepatobiliary injury, especially cholestasis and biliary effects and shows high sensitivity with a low false negative rate in humans (Sheehan and Haythorn, 1979). Rat GGT is expressed in a tissue-specific manner (Chobert et al., 1990). Although the rat GGT assay detects bile duct hyperplasia and necrosis (Leonard et al., 1984), it is considered a less reliable assay compared to GGT enzymatic measurements made in other species.

3.4. Bile acids

Bile acids functionally contribute to the catabolism and elimination of cholesterol; are the primary determinant of bile flow; regulate pancreatic secretions; and release of GI peptides, and contribute to the digestion and absorption of fat (and indirectly fat-soluble vitamins) in the small intestine. Total bile acids are also implicated in various signal transduction pathways and are elevated with liver injury and functional change (Zollner et al., 2006; Geuken et al., 2004). Serum bile acid levels can be influenced by diet and fasting.

4. Sorbitol dehydrogenase shows potential as a biomarker of hepatotoxicity

Sorbitol dehydrogenase (SDH) catalyzes the reversible oxidation-reduction of sorbitol, fructose, and NADH. It is widely distributed in tissues throughout the body, though it is found primarily in the cytoplasm and mitochondria of liver, kidney, and seminal vesicles. It is a specific indicator of acute hepatocellular injury in rodents and has reported value in humans (Khayrollah et al., 1982).

4.1. Liver injury enzymatic activities correlate broadly with general types of liver injury

Fourteen serum biochemical assays were evaluated to predict the presence of hepatic necrosis induced by carbon tetrachloride (centrilobular necrosis), allyl alcohol (periportal necrosis), and 1-naphthylisothiocyanate (ANIT) (biliary duct necrosis) in rats (Carakostas et al., 1986). The GLDH, SDH, and ALT enzymatic assays showed the most diagnostic value of the 14-marker set. The GLDH and ALT activity assays provided the highest diagnostic value for the ANIT and allyl alcohol-induced lesions (Carakostas et al., 1986). The individual assays did not appear to be highly specific for distinguishing between types of hepatic damage from different treatments.

4.2. Translation of biomarker development for SDH

Because SDH activity is a marker of liver toxicity that reportedly translates to dog and rhesus monkey, the development of a stable and improved SDH assay that is commercially available is of interest to industry. SDH enzymatic stability is considered somewhat variable amongst users and was shown to be stable for less than three days when serums were frozen (Horney et al., 1993). To assess effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on liver function, adult male rhesus monkeys were treated with a single oral dose at increasing dosages (Seefeld et al., 1979). Each monkey was used as its own control while ALT and SDH activity were measured at regular intervals for 4 weeks before and 17 weeks after treatment. Serum SDH and ALT activities, but not GGT activity, were elevated in all

TCDD-treated animals. Light microscopy of the livers of TCDD-treated monkeys revealed fatty infiltration with minimal hepatocellular necrosis (Seefeld et al., 1979). In another study, groups of beagle dogs were fed methidathion at increasing dosages. There were no deaths nor adverse clinical signs and treatment-related effects and liver cholestasis was observed in all dogs treated with mid- and high-dietary levels of methidathion (Chang et al., 1992). Statistically significant and moderate increases in serum bile acids and enzyme activities (ALT, SDH, and ALP) were similarly observed in both female and male dogs treated with mid- and high-dietary levels of methidathion. Thus, despite potential technical limitations, SDH is a liver toxicity biomarker that functions across preclinical species and performs comparably to other liver enzymatic activities. It remains to be determined whether serum SDH activity adds value to ALT activity in a large qualification study.

5. Glutamate dehydrogenase activity in acute hepatotoxicity

5.1. Glutamate dehydrogenase activity (GLDH) is a sensitive enzymatic serum marker of liver toxicity

GLDH is present in mitochondria and has a role in oxidative deamination of glutamate. In rats, GLDH is found primarily in liver with lesser amounts in kidney; thus, serum activity originates almost solely from liver. Similar to ALT, GLDH activity increases with hepatocellular damage (O'Brien et al., 2002; Giffen et al., 2003). Plasma activities of ALT, GLDH, AST, SDH, and ALP were compared across several types of liver injury in rats, including partial hepatectomy, exposure to methapyrilene, dexamethasone, cyproterone, isoniazid, lead nitrate, or Wyeth-14643 (O'Brien et al., 2002). Compared to plasma ALT enzymatic increases, GLDH elevations were of greater magnitude (up to 10-fold greater), and persisted longer after treatment. In addition, unlike transaminases, GLDH activity is not inhibited by compounds that interfere with pyridoxal-5′-phosphate, such as isoniazid and lead (O'Brien et al., 2002). Serum GLDH activity is more liver-specific than transaminases and is not substantially affected by skeletal muscle damage. Serum ALT activity was induced by cyproterone and dexamethasone unlike GLDH activity (O'Brien et al., 2002). As a result of increased availability of commercial GLDH reagents globally, investigations could assess the diagnostic performance of this enzyme relative to other standard and newer liver markers.

6. Serum F protein (HPD) as a hepatotoxicity biomarker

6.1. Serum F protein translates as a human biomarker of liver injury

Serum F protein is a 44-kDa protein that is produced in large amounts in liver and small amounts in kidney, while circulating at low serum concentrations in normal human subjects (Oliveira, 1986). Serum F protein was discovered as a mouse antigen from liver extracts that produced precipitating antibody in different mouse strains (Fravi and Lindenmann, 1968). Serum F protein was identified as 4-hydroxyphenylpyruvate dioxygenase (HPD), which is a key enzyme in tyrosine catabolism (Neve et al., 2003). Serum F protein was measured by radioimmunoassay in a variety of human diseases and showed elevations in the serum of patients with hepatocellular damage (Foster et al., 1989). In 87 patients with liver disease, 92% showed increased levels of serum F protein. The serum F protein concentration was a more sensitive and specific marker of liver damage than conventional liver function tests (AST, ALP, GGT activities) and showed a close correlation with the histological assessment of liver damage ($r = 0.86$, $P < 0.001$) (Foster et al., 1989). Serum F protein was measured in patients taking anticonvulsant therapies carbamazepine (CBZ) and phenytoin (PHT) as monotherapeutics, in patients receiving multiple drugs, and in patients taking sodium valproate (VPA) (Callaghan et al., 1994). Serum F protein levels were slightly elevated in 6%, 22% and 13% of patients receiving CBZ, PHT and VPA, respectively. Raised GGT activity levels were higher for both the CBZ (16%) and PHT (44%) groups. Raised ALP activity levels were observed in 13%, 25% and 4% of the CBZ, PHT and VPA groups, respectively. Raised levels of serum F protein in the VPA group and the absence of any associated increases in either GGT or AST activities further supports the suggestion that serum F protein as an indicator of hepatocellular dysfunction associated with anticonvulsant therapy (Callaghan et al., 1994). GGT activity and serum F protein had a correlation coefficient (R value) of 0.56 for CBZ and 0.64 for PHT, both of which were statistically significant. Evidence indicates that serum F protein is not influenced by enzyme induction (Callaghan et al., 1994).

Although serum F protein is produced across a wide variety of mammalian species (Oliveira and Vindlacheruvu, 1987), the correlation between elevated serum F protein and liver histopathologic alterations has not yet been fully elucidated in preclinical animal models (Beckett et al., 1989). Although serum F protein has been

reported as RIA or an ELISA based hepatotoxicity assay, no commercial assay or reagents are available for the general scientific community to further evaluate or qualify. Although limited reagent availability for this analyte has slowed its further evaluation, it is still a potentially valuable biomarker to consider further. Since most of the work on serum protein F has been completed by a single laboratory group, independent confirmation of published findings is recommended before a full qualification is initiated.

7. Glutathione-*S*-transferase alpha as a biomarker of liver toxicity

7.1. GSTα plasma levels can be used as a protein biomarker of liver injury

GSTs are inducible phase II detoxification enzymes that catalyze the conjugation of glutathione with reactive metabolites formed during phase I of metabolism. This action reduces toxicity and facilitates urinary excretion (Beckett and Hayes, 1987). Induction of GST synthesis is a protective mechanism that occurs in response to xenobiotic exposure.

There are four isozymes of GST (alpha, pi, mu, and theta), which are expressed in human and other mammals. The alpha GST class consists of two subunits (dimer) and is expressed in human hepatocytes where it accounts for approximately 5% of the soluble protein (Coles and Kadlubar, 2005). The diagnostic utility of glutathione-*S*-transferase alpha (GSTα) to assess acute hepatotoxicity has been compared to serum ALT and AST activities in studies on both humans and rats. Unlike ALT and AST, GSTα is found in high concentration in centrolobular cells, and therefore is more sensitive to injury in this metabolic zone of the liver. Rats were given a single oral dose of either alpha-naphthylisothiocynate (ANIT), bromobenzene (BrB) or thioacetamide (TAA) that induce marked hepatotoxicity and GSTα elevations corresponded to liver histopathological findings (Giffen et al., 2002). For each compound, fold increases of GSTα compared to matched controls were greater than observations for either serum ALT and AST activities, yet less than those observed with GLDH (BrB and ANIT), SDH (TAA) or total bilirubin and bile acids (ANIT) (Giffen et al., 2002). Although the specificity of GSTα compared to the enzymatic marker values has not yet been fully assessed, GSTα expression is restricted to liver and kidney (Giffen et al., 2002).

In a case series study of nine patients with self-administered acetaminophen overdose, abnormally elevated serum GSTα levels were observed in every case. Moreover, in six cases, GSTα levels and serum F protein (7/9) levels were elevated to twice the reference limit. By contrast, ALT values were within the normal range at admission in all the patients (6 h post-poisoning) except for one who was admitted at 12 h post ingestion. GSTα, serum F protein, and ALT activity all showed increased elevations post-admission, which was consistent with liver biopsies indicating necrosis (Beckett et al., 1989). The five patients with liver damage had GSTα levels greater than 10 μg/L while those without liver damage had GSTα levels less than 10 μg/L. Studies on single dose valproic acid toxicity lead to hemolysis, which interferes with the reliability of the serum ALT and AST activity assays. GSTα levels were used to monitor valproic acid induced hepatotoxicity in rats following daily i.p. injections (500 mg/kg) for 2, 4, 7 10, and 14 days. Significant GSTα elevations occurred with as few as 4 days of treatment and were similarly elevated at days 7, 10, and 14 (Tong et al., 2005). GSTα elevations preceded histopathologic necrosis, although the statistical significance of the elevation at day 2 was not fulfilled. Muscle necrosis is not associated with changes in serum GSTα levels indicating that this marker may be useful in differentiating liver injury from muscle injury (Rees et al., 1995). Low serum GSTα levels in control subjects coupled with high induction of GSTα levels with liver necrosis may aid interpretation of elevated transaminase levels with liver injury.

7.2. Human GSTα polymorphisms could modulate serum GSTα levels

Since single nucleotide polymorphisms effect expression of human GSTα, the lack of GSTα elevations with toxicant treatment might indicate an individual with polymorphism(s) in GSTα. In clinical trials, the monitoring of patients with several biomarkers in addition to GSTα would allow such comparative analyses to be made. The most frequently found polymorphism in human GSTα is in hGSTA1*B, which correlates with reduced liver expression levels since the SP1 promoter element is mutated (Coles et al., 2001). Because the frequency of hGSTA1*B is approximately 40% in Caucasians, 35% in Africans, and 15% in Asians, this polymorphism would have high impact upon the values obtained for participants in clinical trials (Coles and Kadlubar, 2005). Problems in detecting GSTα polymorphisms resulting from variations in coding sequences can be reduced by using polyclonal antisera with broad, but defined specificities. This problem can be analyzed further by comparing changes in subject with their pre-dose values.

8. Arginase I shows promise as a biomarker of liver toxicity

8.1. Arginase I may be an additional ELISA marker for liver toxicity

Arginase is a hydrolase that catalyzes the catabolism of arginine to urea and ornithine. Arginase I is highly liver specific, making it a candidate biomarker that shows higher specificity compared to the liver enzymes (Ashamiss et al., 2004). Serum arginase I was measured in thioacetamide (TAA)-induced acute and chronic liver histopathologic injury in rats in conjunction with serum AST and ALT activities. Arginase I showed the earliest and greatest increase in serum levels among the enzymes tested (Murayama et al., 2007). In humans, liver graft function after transplantation is dependent on ischemia-reperfusion injury, toxicity of drugs (immunosuppression and antibiotics) and transplant rejection. Arginase I was evaluated as a more specific test of liver function compared to traditional serum markers for this model. Serum arginase I peaked at day 1 post liver transplantation and decreased more rapidly than other tests, and showed strong and significant correlation with serum AST and ALT activities (Ashamiss et al., 2004). Since arginase I has been evaluated in a limited set of studies, further preliminary work appears warranted before a larger qualification effort begins. Although some reagents are commercially available, a preclinical arginase I assay is not available for the scientific community to evaluate.

9. Development of novel enzymatic serum liver biomarkers

New safety biomarkers are needed to augment the core of currently accepted indicators of hepatic injury both preclinically and clinically. Genomics, proteomics, and metabonomic analyses are all being utilized for biomarker discovery. Amacher et al. (2005) used proteomic methods to identify serum biomarkers associated with rat liver toxicity or hypertrophy. Four compounds that target the liver through different mechanisms were used to treat rats, and proteomic analysis of sera was used to discover protein biomarkers that correlated with identified hepatic histomorphologic change. Malate dehydrogenase, purine nucleoside phosphorylase, and paraoxanase 1 were amongst the candidate proteins discovered and used for further evaluation (Table 1).

9.1. Malate dehydrogenase

MDH, an enzyme in the citric acid cycle, catalyzes the reversible conversion of malate into oxaloacetate utilizing NAD^+. MDH has been shown to be localized in two cellular compartments, the mitochondria and extra-mitochondria compartment, 10% and 90%, respectively, (Zelewski and Swierczyński, 1991). Generally, the enzyme activity found in the serum is the extra-mitochondria form, but in severe cellular damage, the mitochondrial form can also be detected in serum. The absolute activity in the cytoplasm is greatest in liver, followed by heart, skeletal muscle, and brain (Bergmeyer and Gawehn, 1974). Like ALT, MDH is a periportal enzyme that is released into the serum indicating tissue damage. Serum MDH activity is correlated with both liver and heart injury. Zieve et al., 1985 utilized MDH activity as one biochemical indices of acetaminophen liver injury that coincided with histological evidence of necrosis in rats and Korsrud et al. (1972) reported that MDH activity increases correlated with morphological changes after dosing with thioacetamide, dimethylonitrosamine and diethanolamine. Clinically, Kawai and Hosaki, 1990 showed that MDH activity measurements were more useful in estimating the severity of liver diseases than similar AST measurements and Misra et al. (1991) reported higher levels of MDH activity in cirrhotic patient sera when compared to non-cirrhotic sera.

9.2. Purine nucleoside phosphorlyase

PNP is a key enzyme in the purine salvage pathway. It reversibly catalyzes the phosphorolysis of nucleosides to their respective bases and corresponding 1-(deoxy)-ribose-phosphate. Although the mammalian enzyme is found in a number of tissues, the mean value of PNP activity reported in rat liver is 9.4 U/g, with significantly less reported in heart (0.18 U/g) and muscle (0.59 U/g) (Dwenger and Ivar, 1983). PNP is located mainly in the cytoplasm of endothelial cells, Kupffer cells, and hepatocytes and is released into hepatic sinusoids during necrosis. Ohuchi et al. (1995) cited increases in serum PNP activity after dosing with galactosamine in rats and rat serum activities of PNP were increased earlier compared to ALT following endotoxin treatment that resulted in cellular necrosis (Mochida et al., 1999). Fukuda et al. (2004) utilized PNP activity in combination with glutamine-pyruvic transaminase to evaluate the endothelial cell damage in hepatic sinusoids.

9.3. Paraoxonase-1 is reduced in serum in response to liver injury

PON1 is a high-density lipoprotein (HDL)-associated esterase that detoxifies organophosphates in the liver and protects low-density lipoproteins from oxidative modifications. Unlike MDH and PNP, PON1 is apparently not a leakage enzyme, but rather is released into normal circulation bound to HDL. PON1 is predominantly produced in the liver. However, PON1 activity has been demonstrated in other tissues including kidney, brain and lung (Rodrigo et al., 2001). Decreases in serum PON1 are indicative of tissue damage in liver and are likely due to a reduction in PON1 synthesis and secretion by the liver secondary to a decrease in hepatic PON1 expression (Feingold et al., 1998). Decreases in PON1 activity have been linked to a number of disease states, including atherosclerosis (Ng et al., 2006; Rozenberg et al., 2005), vasculitis (Rozek et al., 2005) and chronic hepatic damage (Ferré et al., 2002; Kilic et al., 2005). Thus, PON1 activity does not appear to show high specificity for liver damage. Nevertheless, a down regulated marker is a extremely valuable addition to a multiple biomarker panel making its evaluation recommended. PON1 activity was also recently identified as a potential biomarker of hepatic injury utilizing proteomic analyses by Meneses-Lorente et al. (2004).

9.4. PON1 polymorphisms in humans

Studies indicate that plasma paraoxonase activity in humans exhibits a polymorphic distribution, with individuals showing a trimodal pattern with either high, intermediate or low paraoxonase activity. Gene frequencies for high or low metabolizers have also been shown to vary among groups of different ethnic or geographical origins (Costa et al., 2002). The molecular basis of the polymorphism has been associated with several mutations (Brophy et al., 2001). It should be noted that in a given population, plasma PON1 activity can vary up to 40-fold (Mueller et al., 1983; Davies et al., 1996; Richter and Furlong, 1999), and differences in PON1 protein levels up to 13-fold are also present within a single $PON1_{192}$ genotype (Furlong et al., 2002; Costa et al., 2003a,b). Measurement of an individual's PON1 plasma activity takes into account all polymorphisms that might affect activity (Costa et al., 2005). This is accomplished through the use of a high-throughput enzyme assay involving two PON1 substrates (usually diazoxon and paraoxon) (Furlong et al., 2002; Josse et al., 1999). Thus, a multiplex assay could help to identify putative PON1 polymorphic activity in conjunction with diagnosing liver injury. Because of polymorphisms, the utility of PON1 activity appears to be greater for preclinical evaluation of hepatotoxicity compared to use in the clinic. Nevertheless, multiplexing technology would enable reduced PON1 values to reflect either liver toxicity or polymorphisms without having to profile the genomes of clinical trial participants.

10. Conclusions

Although serum ALT activity is a highly sensitive biomarker of hepatotoxicity in both preclinical and clinical studies, elevations in the absence of correlative liver histomorphologic changes can be a quandary for compound development. Such ALT elevations can be viewed as false positive signals or potentially prodromal signals. Currently, these modest ALT elevations may necessitate placing a hold upon a developmental compound program(s), which might be lifted if more information became available through newly developed biomarker tools. The biomarkers of hepatotoxicity presented in this review can be compared to ALT enzymatic performance and histomophologic change in preclinical and on occasion, clinical studies in a qualification paradigm. An examination of the current state of hepatotoxicity biomarkers indicates that in conjunction with routine markers, candidates such as serum F protein, arginase I, and GSTα, and the enzymatic markers sorbitol dehydrogenase, glutamate dehydrogenase, paraxonase, malate dehydrogenase, and purine nucleoside phosphorlyase activities may add information to the current assessment of liver toxicity in developmental compound studies. The ELISA markers and the enzymatic markers might be independently adapted to a multiplex measurement platform at significant financial investment and time commitment. Nevertheless, new biomarker tools will aid our evaluation of ALT specificity issues and could also aid our understanding of prodromal ALT signals as predictive markers of liver injury. Since these candidate biomarkers are expressed in both human and rat serum and plasma, we anticipate that the best performing markers will eventually become bridging markers to monitor acute drug-induced liver injury in early clinical trials.

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